Cell Systems
Review

Multiplexed Epitope-Based Tissue Imaging for Discovery and Healthcare Applications

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The study of organs and tissues on a molecular level is necessary as we seek an understanding of health and disease. Over the last few years, powerful highly multiplexed epitope-based imaging approaches that rely on the serial imaging of tissues with fluorescently labeled antibodies and the simultaneous analysis using metal-labeled antibodies have emerged. These techniques enable analysis of dozens of epitopes in thousands of cells in a single experiment providing a systems level view of normal and disease processes at the single-cell level with spatial resolution in tissues. In this Review, I discuss, first, the highly multiplexed epitope-based imaging approaches and the generated data. Second, I describe challenges that must be overcome to implement these imaging methods from bench to bedside, including issues with tissue processing and analyses of the large amounts of data generated. Third, I discuss how these methods can be integrated with readouts of genome, transcriptome, metabolome, and live cell information, and fourth, the novel applications possible in tissue biology, drug development, and biomarker discovery. I anticipate that highly multiplexed epitope-based imaging approaches will broadly complement existing imaging methods and will become a cornerstone of tissue biology and biomedical research and of precision medical applications.

Mammalian cells are never isolated entities in nature. Instead, they are organized in tissues and organs. Organs and tissues, in turn, are assemblies of multiple cell types. In the native context, every single mammalian cell encounters a unique microenvironment, consisting of fluids, matrices, and other cells (Ecker and Steiner, 2004; Gerner et al., 2012; Harnett, 2007; Snijder and Pelkmans, 2011). Even identical cell types display heterogeneity (Altschuler and Wu, 2010; Hanahan and Coussens, 2012; Snijder and Pelkmans, 2011). The processes biomedical research aims to understand are shaped by the interplay between the internal states of cells and the external states in the immediate microenvironment, the surrounding tissue, and the entire organism.

Many research fields now recognize the importance of a cell's microenvironment. For example, stem cell biology can only be understood as a function of the niche in which the stem cell resides (Morrison and Spradling, 2008). During organismal development, a cell's location determines its exposure to tightly regulated growth factors and is key to its future function (Rogers and Schier, 2011). Given the importance of the microenvironment, deregulation of its processes is central to many diseases: type 1 diabetes and cancer are key examples (Hanahan and Coussens, 2012; Lehuen et al., 2010; Polyak et al., 2009). It has recently become clear that the extreme conditions of the tumor microenvironment (e.g., low oxygen and nutrients) support cancer stem cell niches and amplify tumor cell heterogeneity. These processes drive cancer development and resistance to therapy (Hanahan and Coussens, 2012; Polyak et al., 2009; Quail and Joyce, 2013; Trédan et al., 2007).

Although the importance of tissue context is clear, it tends to be destroyed when biological samples are generated and analyzed. A significant part of biomedical research is performed on isolated cells grown in artificial environments. Recently developed organotypic cultures that mimic tissues are an improvement over plastic dishes, but they do not recapitulate the heterogeneity or the multi-cell-type microenvironment of an organism, and model organisms such as mice do not faithfully recapitulate human tissues in health and disease (Shamir and Ewald, 2014). When tissues are studied, these are frequently lysed prior to bulk analysis of thousands to millions of cells, often of different types (Aebersold and Mann, 2003; Lander et al., 2001; Patti et al., 2012; Schena et al., 1995; Soste et al., 2014). Alternatively, tissues can be dissociated into single cells for analyses. This preserves information about cellular individuality, but dissociation perturbs cellular states even under highly controlled conditions. Further, all spatial information, including the tissue morphology and the cell interactions of the microenvironment, is lost (Petit et al., 2013).

So why isn't more life science research performed in human tissues? Manipulating and analyzing tissues is clearly more difficult than analyzing two-dimensional cell cultures (see Challenges from Bench to Bedside). Another reason, I argue here, is that until recently, we did not have the technologies necessary to retrieve enough information from a tissue analysis to perform informative experiments. Importantly, comprehensive experimental results are needed to justify the effort and ethical considerations of working on human tissues. The study of tissues and their complexity automatically demands systems level measurements with single-cell and spatial resolution (Figures 1A and 2). If we want to place a cell into the context of its microenvironment or to define its specific type and state, dozens of cellular components, such as proteins and protein phosphorylation sites, have to be measured simultaneously (Chattopadhyay et al., 2014). Analysis of one or few proteins, phosphorylation sites, or DNA or mRNA molecules does not yield sufficient information

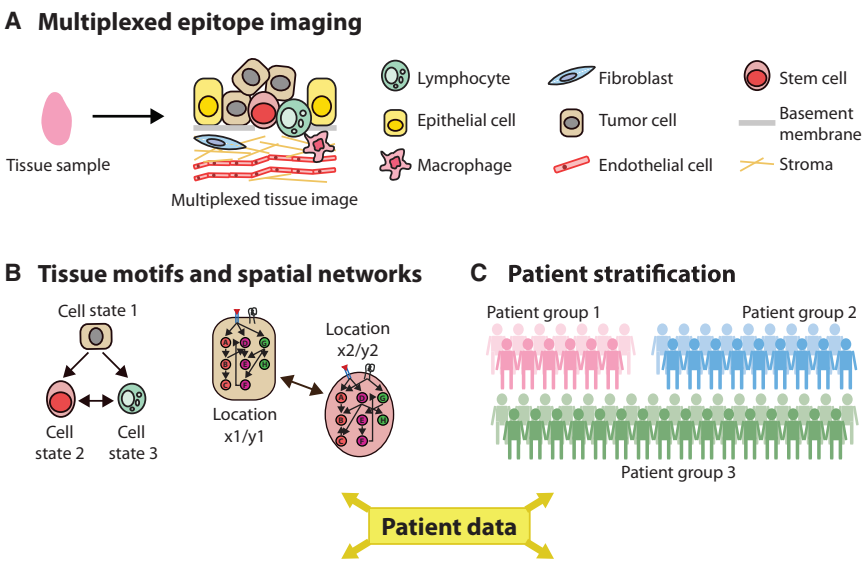

Figure 1. Applications of Highly Multiplexed Epitope Imaging Approaches

(A) Multiplexed imaging provides a comprehensive, spatially resolved view on cell types and their state in tissues.

(B) Statistical and machine learning approaches reveal tissue motifs and enable development of spatial network models.

(C) Correlation of highly multiplexed epitope imaging with clinical data supports biomarker discovery.

(D) Highly multiplexed epitope imaging can be used to identify drug targets and to assess how a drug is distributed in a tissue and the effects it elicits.

(E) Highly multiplexed epitope imaging could be a core tool for future precision medicine applications.

for characterization of tissues and cell microenvironments (Chattopadhyay et al., 2014; Rimm, 2006).

In an ideal scenario, genotype (Navin et al., 2011), transcriptome (Tang et al., 2009), proteome (Bendall et al., 2011), metabolome (Zenobi, 2013), signaling network state (Sachs et al., 2005), and other cellular components would be observed simultaneously in all cells residing in a tissue. This information would enable computational modeling of complex cellular processes and their dependence on both the microenvironment and tissue morphology (Figure 1B) (Angermann et al., 2012; Snijder and Pelkmans, 2011; Snijder et al., 2009). These models could be analyzed in silico to generate hypotheses about biological processes in tissues. Alternatively, patient samples could be put into the context of clinical, personal, and genomic data, facilitating the discovery of disease mechanisms and biomarkers and the development of drugs (Figures 1C and 1D) (Aghaeepour et al., 2013; Bruggner et al., 2014). This Review discusses how new developments in highly multiplexed, epitope-based tissue imaging are an important step in this direction. The Review will also cover the information highly multiplexed epitope-based imaging approaches can provide, how challenges to broadly implement these methods from bench to bedside can be solved and how these imaging approaches can be integrated with orthogonal methods for a multi-layered systems level tissue analysis. Epitope-independent imaging approaches, chromogen and tyramide signal amplification based immunohistochemistry and application to specific biological fields are not discussed. Taken together, this Review will provide a roadmap on how highly multiplexed imaging of tissues could become a cornerstone for future tissue biology and precision medicine applications (Figure 1E).

Tissue Systems Biology Using Novel, Targeted, Multiplexed Imaging Methods

Over the last few years, several new highly multiplexed imaging methods have been developed. These visualize proteins and their modifications at subcellular resolution in tissues (Figure 3) (Angelo et al., 2014; Gerdes et al., 2013; Giesen et al., 2014; Lin et al., 2015; Rimm, 2014; Schubert et al., 2006; Wählby et al., 2002; Zrazhevskiy and Gao, 2013). All of these methods have in common that they measure affinity-bound reagents, mostly antibodies, that specifically target molecules of interest in the tissue (Figures 2 and 3). Because the antibodies used are the same as those used for immunofluorescence microscopy and immunohistochemistry of adherent cells and tissues, respectively, a plethora of antibodies are already available for a broad range of biological and medical applications.

Highly multiplexed epitope imaging approaches rely on either mass cytometry or immunofluorescence microscopy (Figure 3 and Table 1) (Angelo et al., 2014; Gerner et al., 2012; Giesen et al., 2014; Wählby et al., 2002). Immunofluorescence microscopy-based tissue imaging methods can provide images with ~200 nm resolution (Lichtman and Conchello, 2005), which is sufficient to discern subcellular structures. However, multiplexing immunofluorescence microscopy of tissues is challenging and faces fundamental limits. Tissues exhibit auto-fluorescence, an effect amplified by formalin-fixation and paraffin-embedding (Davis et al., 2014; Robertson et al., 2008). Spectral overlap of the fluorescent molecules currently limits multiplexing to seven epitopes and reduces the dynamic range to ~2.5 orders of magnitude (Gerner et al., 2012; Levenson, 2006; Levenson et al., 2015). It has been estimated that ~7 orders of magnitude are needed to simultaneously detect the lowest and highest abundance proteins of a cell (Milo et al., 2010).

Despite these challenges, effective strategies for highly multiplexed epitope imaging using immunofluorescence microscopy have been developed. These rely on serial cycles of tissue staining with up to five antibodies simultaneously (Zrazhevskiy and Gao, 2013), immunofluorescence microscopy, and removal of the antibody signal with heat denaturation, chemicals, or

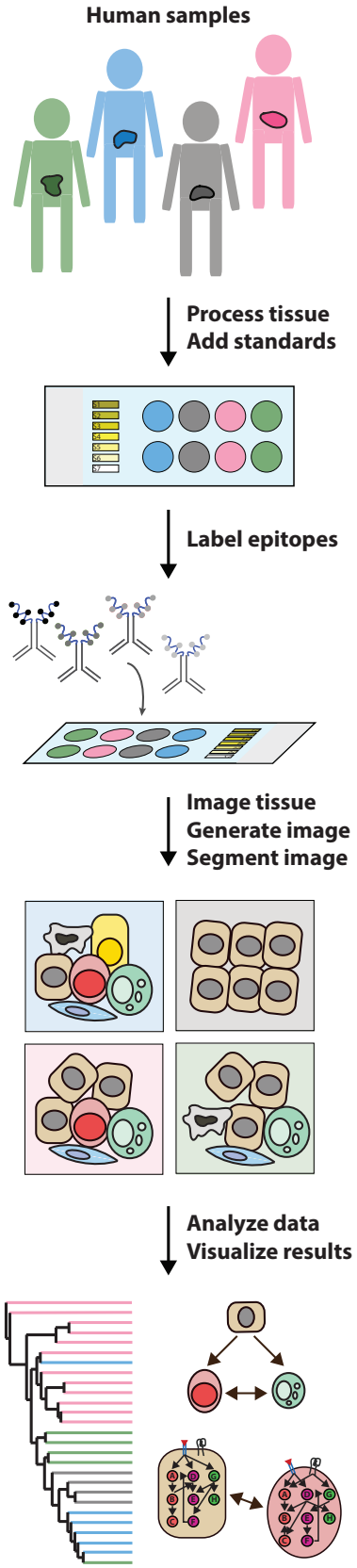

Figure 2. General Workflow for Highly Parallelized Epitope Analysis in Tissues

Tissues are isolated and preserved, then processed for epitope labeling and analyzed by highly multiplexed imaging in parallel with appropriate standards. The thus generated data are used to generate tissue images. After cell segmentation, downstream data analyses and visualization are performed.

photobleaching (Figure 3A; Table 1) (Gerdes et al., 2013; Lin et al., 2015; Schubert et al., 2006; Wählby et al., 2002; Zrazhevskiy and Gao, 2013). Several factors limit the total number of proteins that can be visualized by serial immunofluorescence microscopy. A single cycle to measure up to five antibodies typically takes hours. As the tissue is unprotected and exposed to chemicals over prolonged time periods, changes in antigenicity and signal decay are observed. Furthermore, signal quenching can be incomplete, leading to increased fluorescence background, measurement artifacts, and reduced data quality as the tissue is subjected to cycle after cycle of staining and quenching (Gerdes et al., 2013; Lin et al., 2015; Schubert et al., 2006; Wählby et al., 2002; Zrazhevskiy and Gao, 2013). On the plus side, large tissue areas can be readily analyzed, the approach can be easily implemented using standard immunofluorescence microscopy equipment, complete systems can be purchased, and highly multiplexed epitope imaging by immunofluorescence microscopy is also available as a service.

Mass cytometry-based imaging methods (Angelo et al., 2014; Giesen et al., 2014) overcome many of the difficulties associated with immunofluorescence imaging. In mass cytometry imaging, metal isotopes of a defined atomic mass are used to label antibodies. After simultaneous incubation of the tissue samples with metal-labeled antibodies and binding to the respective epitopes, the isotopes are visualized using mass spectrometry, as I will discuss below. Because non-biological metal isotopes can be used, one advantage of these methods is that the tissue background signal is basically absent. In addition, owing to the abundance sensitivity of the used mass spectrometers, neighboring isotopes can be resolved. As a result, mass cytometry supports much higher multiplexing than does immunofluorescence imaging, with up to 44 and 32 isotope-labeled antibodies demonstrated thus far in cell suspension mode and mass cytometry-based imaging, respectively (Bendall et al., 2014; Giesen et al., 2014). Signal leaking from one antibody channel into another antibody channel resulting in artificial signal is very low at <3% and the linear dynamic range is at least 5 orders of magnitude (Bandura et al., 2009; Levenson et al., 2015). Further, the simultaneous staining of all epitopes with the isotope-labeled antibodies and the subsequent simultaneous detection avoids epitope decay and image registration difficulties, respectively.

Two mass cytometry-based methods were published recently, imaging mass cytometry (Giesen et al., 2014) and multiplexed ion beam imaging (Angelo et al., 2014). In each method, first an instrument, either a laser (Giesen et al., 2014) or an ion beam (Angelo et al., 2014), converts a "two-dimensional" tissue sample (i.e., section) into a stream of particles. Next, a second instrument analyzes the masses of the metal isotopes contained in these particles. Finally, computer software reconstructs a two-dimensional image from the mass data and the known raster positions of the laser or ion beam.

In imaging mass cytometry, a laser ablation system is coupled to an inductively coupled plasma time-of-flight mass

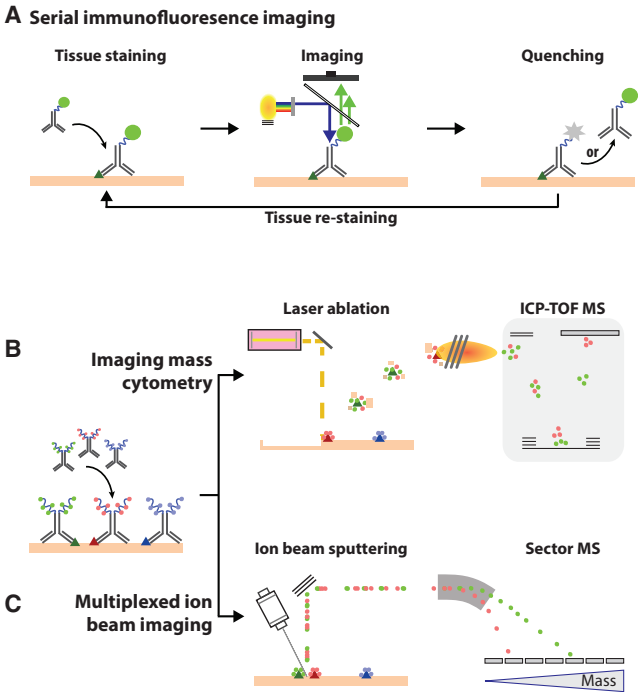

Figure 3. Immunofluorescence versus Mass Cytometry for Highly Multiplexed Imaging

(A) Serial immunofluorescence imaging uses cycles of tissue staining and imaging with one or a few fluorescently labeled antibodies, quenching of the fluorescence by fluorochrome destruction or antibody removal, and re-staining with additional antibodies.

(B) In CyTOF imaging mass cytometry, a tissue is stained with dozens of metal-labeled antibodies simultaneously. A high-resolution laser ablation system is then used to transfer the tissue spot-by-spot into the CyTOF mass cytometer to determine metal isotope content and, therefore, epitope expression.

(C) In multiplexed ion beam imaging, the tissue is stained with ten metal-labeled antibodies simultaneously. A primary ion beam is used to raster over the tissue to generate secondary ions, among them the metal isotopes that were bound to the antibodies. A sector field mass spectrometer is then used to determine the metal isotope content and, therefore, epitope expression in each rastered area. ICP-TOF MS, inductively coupled plasma time-of-flight mass spectrometer. Mass spectrometer, MS.

spectrometer called a CyTOF instrument (Figure 3B; Table 1) (Giesen et al., 2014; Wang et al., 2013). The laser ablates the tissue spot by spot with a resolution of ~1,000 nm, generating particles. These particles are transported into the CyTOF using a gas stream; in the CyTOF, they are atomized and ionized in the plasma ion source. The metal-isotope ion content, and, by inference, epitope abundances and distributions, are determined in the time-of-flight mass analyzer. In this setup, each laser shot is akin to a pixel of a tissue image (Giesen et al., 2014).

In multiplexed ion beam imaging, secondary ion mass spectrometry is used (Angelo et al., 2014) (Figure 3C). A duoplasmatron ion source is used to generate the primary (oxygen) ion beam, which is rastered over the tissue with a lateral resolution of ~200 nm; these primary ions remove antibody-bound metal isotopes as ions. These ions generated by the primary ions are called secondary ions. The secondary isotope ions are analyzed in a magnetic sector mass analyzer (Figure 3C; Table 1) to infer the presence and abundance of epitopes in the tissue (Angelo et al., 2014).

The two mass spectrometry-based approaches have complementary characteristics (Table 1). Due to the simultaneous mass analysis in the time-of-flight analyzer, imaging mass cytometry has the potential to measure the abundances of up to 135 isotope-linked antibodies simultaneously; currently 32 isotopes are routinely used as labels on antibodies (Giesen et al., 2014). For sector field mass analyzers the number of detectors determines the simultaneous mass channels; seven are currently employed concurrently in multiplexed ion beam imaging (Angelo et al., 2014); however, in multiplexed ion beam imaging only a thin layer (20–50 nm) of the tissue is removed, and iterative scanning of the same tissue with a different detector configuration can increase the number of analyzed antibodies to at least 10 (Angelo et al., 2014). Imaging mass cytometry is highly quantitative due to the absence of matrix effects; that is, ion generation does not depend on the chemical composition of a tissue area. By contrast, in multiplexed ion beam imaging, matrix effects exist and make quantification more challenging. Finally, primary ion beam imaging coupled to a sector field mass spectrometer is highly sensitive with a detection limit of ~25–125 antibodies per pixel at 200 nm lateral resolution compared to ~100–500 antibodies per pixel for imaging mass cytometry at 1,000 nm lateral resolution (assuming the same ablation depth) (Table 1). The throughput of both approaches currently is low; a 1 mm^2 field of view takes approximately eight hours to analyze (Angelo et al., 2014; Giesen et al., 2014; Wang et al., 2013). Sources of noise exist: Not all isotopes for antibody labeling are 100% pure and therefore signals can overlap among mass channels according to the impurity, necessitating a smart antibody panel design (Bandura et al., 2009; Bendall et al., 2012).

Laser ablation systems geared for tissue analysis with the CyTOF instrument are becoming commercially available, which will enable broad adaptation of the technique. Sector field secondary ion mass spectrometry instruments are common in academic institutions, but the ultra-high resolution nanoscale secondary ion mass spectrometry instruments employed for multiplexed ion beam imaging are rare.

Imaging mass cytometry and multiplexed ion beam imaging are in an early stage of development, and all performance parameters must be improved to enable broad adoption. Projecting recent developments into the very near future, I expect that acquisition speeds will increase by at least one order of magnitude. Adapting existing chemistries of metal nanocrystal and nanoparticle synthesis to pure isotopes and antibodies will facilitate the development of novel reporters with higher numbers of metal atoms to increase sensitivity, resolution (which are functions of each other), and the number of epitopes that can be measured simultaneously (Lou et al., 2007).

Comprehensive Information from Proteins to Cells to Environmental Context

Once a tissue has been analyzed by highly multiplexed epitope imaging, three levels of information can be retrieved. On the first level, the expression of epitopes of interest, which "mark," that is, report on, cell types and biological processes, can be analyzed without single-cell assignment (Figure 4). This provides information describing which cell types and biological processes are present. Furthermore, the spatial distributions of these markers can be analyzed to relate the biological activities they

Table 1. Highly Multiplexed Epitope Imaging Approaches

	Serial Immunofluorescence Microscopy	CyTOF Imaging Mass Cytometry	Multiplexed Ion Beam Imaging
Resolution	~200 nm	~1,000 nm	~200 nm
Limit of Detection (Number of Antibodies per Pixel)[a]	20–200	100–500	25–125
Simultaneous Readouts	1–5 per cycle	32[b]	7 per scan
Repeat Analysis of Same Section with Different Antibodies	Yes	No	Yes
Maximum Epitopes Multiplexed	90	32	10
Throughput[c]	hours per cycle/500 mm^2	~14 hr/mm^2	~8 hr per scan/mm^2

All numbers are published or estimates based on literature for tissue analysis.
[a]All numbers are estimates. These numbers depend on the fluorophore and autofluorescence of the analyzed tissue area for fluorescence microscopy and the thickness of the removed tissue for both mass cytometry based methods.
[b]Current instrumentation allows for 135 simultaneous channels, but the approach is currently limited by the available reagents.
[c]Instruments and reagents under development will have markedly improved performances enabling routine application in research and clinical applications.

report on to the morphological features of tissues. Compared to previous tissue analyses methods, highly multiplexed epitope imaging yields a comprehensive view of tissue composition and marker distribution.

On the second level, the tissue image can be segmented into features that correspond to single cells (Figure 4, see also Challenges from Bench to Bedside) (Carpenter et al., 2006; Eliceiri et al., 2012; Gerner et al., 2012). Using algorithms like spanning tree progression of density normalized events (Qiu et al., 2011), t-distributed stochastic neighbor embedding (Amir et al., 2013; van der Maaten and Hinton, 2008), and others (Aghaeepour et al., 2013), cell types and cell heterogeneity can be analyzed in an unbiased manner. Cells visualized by highly multiplexed epitope imaging can be further segmented into subcellular compartments, and biological activities can be assigned to each compartment based on the presence or absence of markers (Carpenter et al., 2006). This is relevant to understanding the biology of both health and disease, as changes in cell composition and delocalization of biological activity drive many pathologies. Finally, marker correlations in the single-cell data can indicate interdependent biological processes (Sachs et al., 2005).

On the third level, a spatially resolved view of the tissue allows each cell type and its functional state to be put into environmental context (Figure 4) (Ecker and Steiner, 2004; Gerner et al., 2012; Harnett, 2007; Snijder and Pelkmans, 2011; Snijder et al., 2009). Cell-types, cellular processes such as signaling or apoptosis, and subcellular location of biomolecules can be placed within their environmental context. For example, if signaling molecules, receptors, and downstream pathways are analyzed, cell-cell communication and signal propagation through many cells can be studied. When the function of a tissue (e.g., the secretion of a hormone) can be quantified, the function can be related to cell types, states, and "neighborhoods," that is, regions of the tissue that share common features. The ability to perform highly multiplexed analysis of functions in single cells of defined type in relation to the environment and to tissue-level function is unique to highly multiplexed epitope imaging approaches. It enables truly systems-level biological, biomedical, and clinical applications as I will describe in Applications for Precision Medicine and Systems Analysis of Disease.

Challenges from Bench to Bedside

Highly multiplexed epitope imaging of tissues is becoming widespread and has the potential to profoundly impact life science research and clinical practice. In a typical workflow, a tissue from a donor, patient, or a model system is isolated, preserved, and then processed for staining using affinity reagents (Figure 2). The tissue is then analyzed by highly multiplexed imaging in parallel with appropriate standards; these standards enable normalization of the measured signal over time among samples and to determine molecular copy numbers (Figure 2). Subsequently, these data are processed to generate the image, the three layers of information are extracted, and downstream data analyses and modeling are performed (Figure 2) (Gerner et al., 2012; Giesen et al., 2014; Snijder and Pelkmans, 2011). Although the protocols for highly multiplexed epitope imaging are readily implemented and guidelines for data storage and analysis exist for related methods (Carpenter et al., 2006; Eliceiri et al., 2012; Lee et al., 2008), each step of the workflow poses challenges for method adaptation and reproducibly of the approaches (Table 2). Several issues must be overcome for routine use of these methods in biomedical research and in a clinical setting. Many of these challenges, as discussed below, are *independent* of the imaging technologies themselves, and common to all methods aimed at tissue analysis. None are insurmountable (Table 2).

Standard Operating Procedures for Tissue Isolation and Preservation

Tissue analyses will only be as good as the samples used. When tissues are removed from an organism, cells respond to the absence of specific nutrients and oxygen transport the moment blood supply to the tissue is restricted (Spruessel et al., 2004). This restriction, called ischemia, results in changes in protein phosphorylation levels within minutes and slower changes in protein levels over hours (Bray et al., 2010; Gündisch et al., 2015; Pinhel et al., 2010; Spruessel et al., 2004). Human samples are often stored temporarily at 4°C, and eventually most tissues are preserved in formalin. Cooling and formalin fixation are slow processes (formalin diffuses into tissue at about ~1 mm per hour) (Thavarajah et al., 2012) that generate artificial biochemical gradients from the tissue surface to the core (Gündisch et al., 2015). The ratio of formalin to tissue mass and the crosslinking

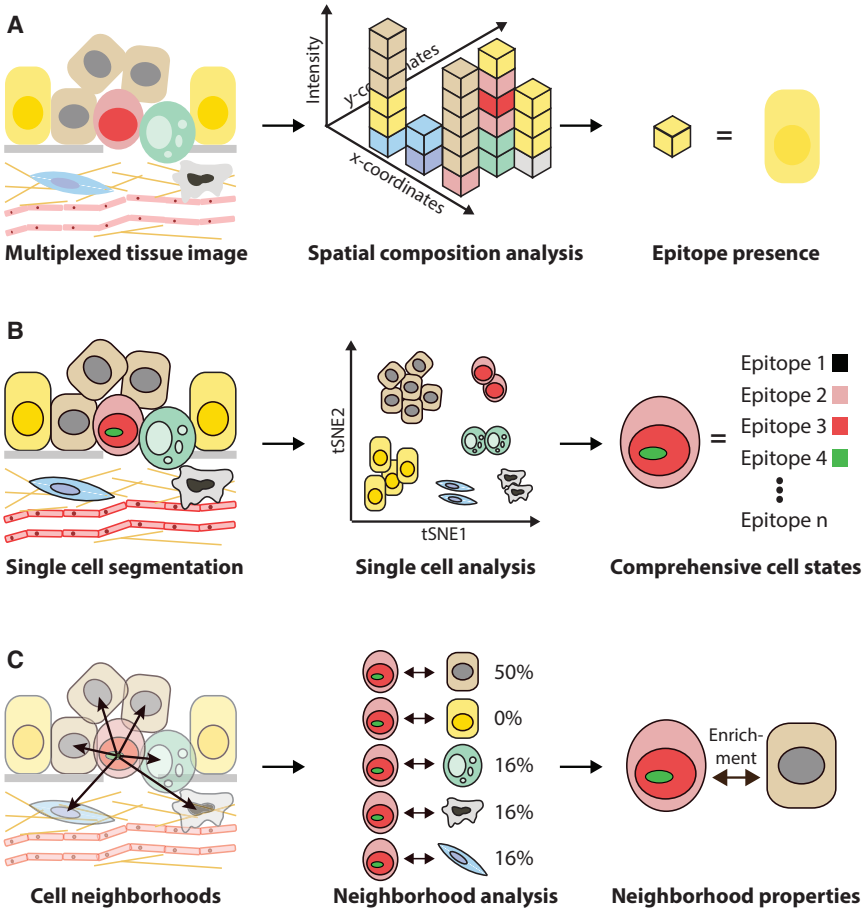

Figure 4. Information Content of Highly Multiplexed Images

(A) Epitopes expressed in a given region of the sample of interest can be detected.

(B) In combination with single-cell segmentation, complex cell phenotypes can be inferred and analyzed.

(C) Exploiting spatial resolution, cell-cell interactions and cell microenvironments can be studied.

Pieces of tissues or stem cells can be grown in extracellular matrix to form organoids (Hynds and Giangreco, 2013; Shamir and Ewald, 2014), miniature three-dimensional morphological representations of the tissue of origin. The cells present in organoids are heterogeneous (Shamir and Ewald, 2014), and organoid models have been established for disease-relevant organs (Sato et al., 2009; Shamir and Ewald, 2014). Donor-specific organoids can be generated, and it has been suggested that this may be of use in precision medicine (Shamir and Ewald, 2014; van de Wetering et al., 2015).

Organoids recapitulate organizational principles of tissues, but not their complexity. Cells of immune and stromal origin and other tissue components such as the extracellular matrix and vessels are absent. To overcome this limitation, slices of organs can be cultured ex vivo (Collins et al., 2000; Vaira et al., 2010). Here, viable tissues are sectioned at a thickness of up to 1 mm and are cultured on synthetic membranes (Hardman et al., 1993). Whereas morphology and cell composition are preserved initially, tissue slices typically show changes in transcription profile, cell content, and cell neighborhoods within days (Vaira et al., 2010).

Finally, human tissues can be grown in immunocompromised mice as xenografts. This approach allows the host physiology to effect the donor tissue (Morton and Houghton, 2007). As the host organism does not match the donor, the environment is artificial (Morton and Houghton, 2007; Tentler et al., 2012).

times of the fixation reaction affects results of downstream analyses but are rarely controlled in clinical settings (Gündisch et al., 2015; Neumeister et al., 2014; Pinhel et al., 2010; Spruessel et al., 2004). Shock freezing preserves cell states, but frozen tissues are difficult to handle, often lose histological and cytomorphological structure, and are prone to artifacts when analyzed using antibodies (Shi et al., 2008). Not only is it challenging to preserve a tissue in a state that is representative of the in situ biology, but procedures applied in clinics are highly variable. Many hospitals lack meticulous standard operating procedures for tissue handling (Robb et al., 2014). To make analyses comparable between hospitals, accurate standard operating procedures that describe every step of tissue handling and processing should be widely adopted. Standardized processing would improve data quality and would enable accounting for confounding factors by means of statistical methods (Robb et al., 2014).

Model Systems of Tissue Biology

Even though they do not necessarily reflect in situ biology, model systems, including organotypic cultures such as organoids, tissue slice cultures, and mouse models, are essential (Shamir and Ewald, 2014). These model systems can be readily manipulated with the existing arsenal of molecular biology methods such as genome editing and reproducibly generated. Both aspects are keys to enable deep mechanistic insights into tissue biology and disease. Ideally, these models can be used to confirm observations made on human samples.

Epitope Labeling with Affinity Reagents

Universally, in highly multiplexed imaging, data quality is a direct function of antibody quality. Antibodies have application-dependent performances and optima (Bradbury and Plückthun, 2015). For example, for formalin-fixed, paraffin-embedded tissues the epitope must be retrieved—that is the chemical modifications of the epitope introduced by the reaction with formaldehyde must be reversed—and the optimum retrieval conditions vary from antibody to antibody (Howat et al., 2014; Pauly and Hanack, 2015). In addition, antibody preparations, even when purchased from the same vendor, can be irreproducible and unspecific for the claimed epitope (Bradbury and Plückthun, 2015). For these reasons, the specificity of every antibody must be confirmed. This is usually done by benchmarking the antibody's performance using samples known to express and not to express

Table 2. Factors to be Implemented for Highly Multiplexed Tissue Analysis from Bench to Bedside

Challenge	Requirements	Current State	References
Samples			
Standardize tissue processing	Broadly adopted standard operating procedures for all steps of tissue processing to enable comparative studies. Protocol every step of tissue processing to account for confounding factors in downstream analyses. Employ protocols that avoid artifacts by ischemia, cooling and formalin fixation.	Standard operating procedures for tissue processing vary between hospitals. Deviations from the standard operating procedures are frequently not recorded. Procedures to preserve tissue biology often absent.	Gündisch et al., 2015; Robb et al., 2014; Spruessel et al., 2004; Thavarajah et al., 2012
Develop model systems recapitulating human tissue biology	Recapitulate cellular and cell-type heterogeneity, and tissue function in a faithful and reproducible manner.	Organoids; tissue slice cultures; mouse models. Development of model systems is advancing rapidly, no model system reflects in situ biology though.	Collins et al., 2000; Sato et al., 2009; Shamir and Ewald, 2014; Tentler et al., 2012
Measurement			
Develop reproducible affinity reagents for all epitopes informative of cell type and cellular processes	Each affinity reagent needs to be reproducible and specific for a given epitope. Application independent performance.	For some epitopes affinity binders that fulfill all requirements exist. Initiatives to generate reproducible affinity reagents for most proteins are ongoing.	Bradbury and Plückthun, 2015; Howat et al., 2014; Pauly and Hanack, 2015; Uhlén et al., 2015
Normalize data over time and between laboratories	Standards to normalize for instrument variation, and reference samples with known antibody binding capacity and epitope numbers for calibration.	Standards to normalize for instrument performance are available. Standards for anti-body binding capacity and copy number calibration are lacking.	Brownridge et al., 2011; Carvajal-Hausdorf et al., 2015; Gratama et al., 1998; Longerich et al., 1996
Analysis			
Perform single-cell segmentation of tissue images without errors	Epitopes informative of cell boundaries in all tissue settings. Segmentation algorithms using all information contained in the multiplexed images and geared for the complications of the tissue setting.	Algorithms are under development; first algorithms exploiting multiplexed image data for segmentation are available.	Carpenter et al., 2006; Eliceiri et al., 2012; Schüffler et al., 2013, 2015
Analyze and visualize data	Computational tools for highly multiplexed data that enable biologists to intuitively analyze cell states, cell neighborhoods, spatial networks, and associations to clinical data.	Computational tool boxes are under development. Approaches such as tSNE, Phenograph and Citrus can be readily applied to analyze image derived single-cell and neighborhood data. Novel algorithms to study tissue motifs and cell neighborhood (networks) need to be developed.	Bruggner et al., 2014; Eliceiri et al., 2012; Mair et al., 2016; van der Maaten and Hinton, 2008
Store multiplexed image data in a common format, independent of used platform	Image data, standard data, and meta data need to be stored in a common file format.	imzML format for mass spectrometry data exists. Formats for microscopy-based imaging are established as well. Community needs to agree on a standard.	Eliceiri et al., 2012; Lee et al., 2008; Schramm et al., 2012

the epitope. Then, antibody performance is optimized for the intended sample type. These experiments require systematic variation of antibody concentration and variation of sample processing parameters (Howat et al., 2014; Pauly and Hanack, 2015). For a panel with dozens of antibodies such validation and optimization is a tedious task. Some affinity reagents (including monoclonal antibodies, aptamers, and affimers) are deeply validated and can be reproducibly generated, but such high quality reagents must be developed for proteins and phosphorylation sites indicative of all key cellular processes and cell types to allow truly systems-level, multiplexed imaging.

Standardization and Molecular Copy Number Quantification

To support comprehensive comparison of data over time and across laboratories and to support mathematical modeling, one must accurately quantify the amount of bound antibody or the expression of an epitope (Carvajal-Hausdorf et al., 2015). Currently, only relative intensities of epitopes and percentages of cells expressing an epitope are typically inferred from a tissue image (Carvajal-Hausdorf et al., 2015; Gerner et al., 2012).

To enable quantitative analyses, we need reproducible standards that can be analyzed on all highly multiplexed epitope imaging platforms. Three levels of standardization can be envisioned. First, fluorophore- and metal-containing standards could be measured with all samples to enable normalization of the performance of the instrumental set-up between measurements and laboratories; metal standards are available from the National Institute of Standards and Technology (Longerich et al., 1996). Second, the antibody binding capacity per pixel could be determined (Gratama et al., 1998). Areas of defined titrations of known antibody binding capacities could be co-processed and analyzed with tissues to determine per pixel antibody binding. Third, absolute epitope copy numbers could be determined (Brownridge et al., 2011; Gratama et al., 1998). Absolute quantification of cellular molecules enables an accurate comparison of data generated between labs, independent of the used reagents and methods. Absolute quantification is independent of experimental workflows and compensates for nonspecific antibody binding, unknown antibody binding site occupancy, epitope accessibility, antibody labeling efficiency, and antibody staining concentration. Cell lines (and tissues) with known epitope copy numbers (e.g., determined by protein mass spectrometry [Brownridge et al., 2011] or via absolute quantified fluorescent protein labels [Ghaemmaghami et al., 2003]) could be stored (e.g., as formalin-fixed, paraffin embedded samples) and processed in parallel with all samples during highly multiplexed epitope imaging. Comparison of the tissue single-cell signals with the parallel-processed absolutely quantified calibration cells would enable copy number determination.

Absolute quantification would also facilitate identification of novel features in the context of cell neighborhood environments and would expand the systems view on a tissue in several ways. First, the levels of individual nodes within a network could be determined. Second, the stoichiometries of proteins (complexes) and protein phosphorylation site occupancies could be computed per pixel. Third, low copy-number effects that may render biological process noisy could be inferred within individual cells and concentration thresholds for switching between cellular states could be inferred from single-cell-resolved population-level data. Fourth, if time series data were available, kinetic rates of cellular reactions such as the speed of protein phosphorylation events executed by a kinase could be gleaned.

Image Processing and Single-Cell Segmentation

To study cell types and interactions, one needs to demarcate individual cells on the tissue image. This is achieved through image segmentation, typically by assuming that epitopes located in/at a cell membrane define a cell's boundary (Carpenter et al., 2006). Segmentation is challenging, and none of the available algorithms for tissues are completely satisfactory for three major reasons that reflect the complexity of tissues (Schüffler et al., 2013). First, cell sizes and cell shapes can vastly differ among immune, stromal, and tumor cells, and cells can be too close together to be demarcated. Second, images are typically based on 2- to 5-μm-thick sections of a tissue, only partially and randomly representing cell volumes. Consequently, the nucleus (which is often used as a feature for segmentation) can be missing, the two-dimensional cell shape might not represent the three-dimensional one, and cells can lie over each other within a section. Third, membrane markers typically used for segmentation can be located anywhere in diseased tissues and can be at different locations even for subtypes of the same disease. In aggregate, these complexities tend to produce certain types of errors. For example, the failure to indicate overlapping cells can result in erroneous cell-type detection (Schüffler et al., 2013, 2015). New algorithms that exploit all information contained in the highly multiplexed tissue images and use all epitopes for segmentation will likely mitigate these errors. An additional improvement could be achieved by generating and segmenting three-dimensional representations of the tissue based on analysis of serial sections.

Data Analysis and Visualization

Many data handling and analysis pipelines for tissue image analysis exist; most, however, were developed for immunofluorescence microscopy of adherent cells (Eliceiri et al., 2012). The pipelines include ImageJ, OMERO, Cell profiler (Carpenter et al., 2006), Tmarker (Schüffler et al., 2013), Definiens, and others (Eliceiri et al., 2012), but none are geared to exploit the three levels of systems information offered by highly multiplexed epitope imaging of tissues. Efforts are ongoing to address this need.

High-dimensional single-cell data are subject to the ''curse of dimensionality''—that is, additional data dimensions result in the need for exponentially more data to avoid a sparse data distribution. Such sparse data distributions hinder development of predictive mathematical models and limit the ability to draw reliable conclusions. By adding the dimension of space, the number of dimensions for each single cell is much larger: location in the tissue, the cell shape, members of the direct and extended cell neighborhood and their (high parameter) states, and many more parameters can be obtained. This problem of high-dimensional data can be approached by generating very large datasets, but often this solution is not practical. Due to the large degree of dependency and informational redundancy between the features that are extracted from the images (e.g., some epitopes are always co-expressed in the same cell type or cell size and shape correlate with cell type defining epitopes), the data can be usually represented with fewer dimensions. To achieve this, data dimensionality reduction approaches can be

used. The underlying assumption is that the high-dimensional data can be represented in a low(er)-dimensional space without loss of important information. These issues have been reviewed elsewhere (Amir et al., 2013; Mair et al., 2016; Pearson, 1901; van der Maaten and Hinton, 2008). Some data dimensionality reduction approaches perform better than others, and low-dimensional visualization and analysis are often hard to interpret from a biologist's perspective. The most suitable approach for data dimensionality reduction to analyze highly multiplexed single-cell resolved imaging data and cell neighborhoods has not yet been determined.

Data Standards and Dissemination

Minimum information standards for sharing of highly multiplexed epitope imaging data should be established (Eliceiri et al., 2012), akin to the MIFlowCyt (Lee et al., 2008) standard for flow cytometry. Such standards will facilitate data reproduction and meta-analyses. To facilitate this, a common file format should be defined. For mass spectrometry imaging data, the imzML format, which is based on the Human Proteome Organization mzML format, is used (Schramm et al., 2012). Here image data are stored as a binary file and meta-data (e.g., instrument settings and experimental parameters) are stored as controlled vocabulary in an XML file (Schramm et al., 2012). Antibody information, experimental variables, tissue source, patient and clinical information, and calibration standards must all be captured.

The establishment of publicly accessible and free databases in which to store these files would encourage meta-analyses and algorithm development. One example of a database for storage of epitope-based tissue image information is the Human Protein Atlas (Uhlén et al., 2015). As this example demonstrates for immunohistochemistry data (Kampf et al., 2012; Pontén et al., 2008; Uhlén et al., 2015), a resource of data generated using common standards and stored in a common format with annotated experimental and clinical information would allow researchers to exploit thousands of samples to answer questions about tissue biology, to identify reliable biomarkers, and to serve as a baseline for precision medical applications.

Moving Beyond Protein Measurements

Most highly multiplexed epitope imaging methods detect proteins and their modifications. The highly multiplexed capabilities discussed here offer the exciting possibility of combining highly multiplexed epitope imaging with genomic (Navin et al., 2011), transcriptomic (Tang et al., 2009), metabolomic (Zenobi, 2013), enzymatic activity (Van Noorden, 2010), and live cell readouts (de Jong et al., 2014). DNA and mRNA can be analyzed in tissues using fluorescence in situ hybridization (Crosetto et al., 2015). Different approaches for tissue fluorescence in situ hybridization are commonly used including branched fluorescence in situ hybridization (Wang et al., 2012) and padlock probes (Nilsson et al., 1994). Developments such as in situ RNA sequencing and in situ PCR may allow generation of single-cell expression and genomic profiles, respectively. These techniques could be especially informative when used in conjunction with the epitope readouts at the protein level (Chen et al., 2015; Janiszewska et al., 2015; Ke et al., 2013; Lee et al., 2014). Further, highly multiplexed epitope imaging could be combined with measurement of enzymatic and metabolic activity in live tissues and tissue cryostat sections (Baruch et al., 2004; Van Noorden, 2010). In one strategy for measuring enzymatic activity, the enzyme is exposed to synthetic substrates, and the chemistry the enzyme catalyzes converts synthetic substrates into chromogenic or fluorogenic products. These probes have been developed for many enzyme classes. Alternatively, activity-based probes can be used (Cravatt et al., 2008; Hunerdosse and Nomura, 2014). In such approaches, synthetic, reporter-tagged molecules are designed to covalently react with the active form of an enzyme. Molecules that react with proteases, phosphatases, and kinases have been already developed (Cravatt et al., 2008; Hunerdosse and Nomura, 2014).

Highly multiplexed epitope imaging based on fluorescence can be readily adapted to accommodate antibody-based detection and the above-mentioned non-antibody detection methods. The main challenge here is that the currently used workflows are not optimized to allow readout of genomic, transcriptomic, metabolomic, and enzymatic activity simultaneously with epitope level information. In addition, for the mass cytometry-based highly multiplexed epitope imaging approaches, both reagents and protocols need to be adapted. For example, fluorophores could be replaced by metal reporters for readout by mass cytometry or tissues could first be analyzed by immunofluorescence microscopy using existing fluorophore-based readouts and subsequently by mass cytometry methods. Ultimately the latter will be the method of choice given the ease of coupling immunofluorescence microscopy with highly multiplexed epitope-based mass cytometry imaging.

Currently, a fundamental limitation to highly multiplexed epitope imaging is the fact that it is conducted on frozen or fixed tissues. If live-cell highly multiplexed imaging approaches were developed, correlations between time-resolved processes and a systems level readout could be directly observed. This approach would either rely on genetics approaches to express many process- and cell-type-specific reporters or on the transfer of (affinity-based) reporter reagents for multiplexed imaging into living cells. For human tissues, mostly the latter method would be applicable. Not all cells of a tissue take up reporter/affinity reagents, and the reagents themselves strongly affect processes in living cells. Further, fluorescence-based methods would be subjected to all fundamental limits discussed above, and mass cytometry-based analysis is destructive.

Alternatively, tissues could first be studied using existing live cell imaging approaches, and at informative time points, the tissue could be fixed and analyzed by the highly multiplexing imaging approaches. Such snap-shot imaging data could then be overlaid on the live cell data, individual cells could be registered, and the time-resolved and snap shot data analyzed together. The current method of choice for live-tissue analysis is two-photon microscopy, which allows high-resolution imaging to a depth of 1 mm (standard confocal immunofluorescence microscopy is limited to ~100 μm) (Denk et al., 1990; Helmchen and Denk, 2005). Excitation is necessary for fluorescence imaging of live tissue, and this can cause autofluorescence, photobleaching, and phototoxicity (Takai et al., 2015). These confounding effects have been avoided through the development of methods based on enzymes (e.g., luciferase) that produce light upon substrate exposure (Takai et al., 2015). Luciferases can be used to monitor gene expression, enzymatic activity (such as protein phosphorylation [Ishimoto et al., 2015]), and

motility and to label specific cellular compartments (Lee et al., 2015). Bioluminescence imaging can also be applied to visualize processes in tissues located centimeters deep in an organism. Methods for deep tissue visualization, also including positron emission tomography, single photon emission tomography, and fluorescence-mediated tomography, typically lack single-cell resolution, however (de Jong et al., 2014). Coupled to highly multiplexed imaging, all of these approaches will allow better understanding of time-resolved processes and will yield systems level molecular readouts.

Integrating Epitope Imaging with Other 'Omics Data

Highly multiplexed epitope imaging can also be combined with other methods and data offering a systems view on tissues including genotype (Lander et al., 2001), transcriptome (Schena et al., 1995), proteome (Aebersold and Mann, 2003; Soste et al., 2014), and metabolome (Patti et al., 2012; Zenobi, 2013). Three possible applications include selection of the most informative epitopes, demarcation of interesting tissue areas, and direct spatial 'omics measurements.

Highly multiplexed epitope imaging is still limited in the number of epitopes it can measure, therefore, utility depends on the measurement of the most informative readouts, which are typically chosen in the light of prior knowledge. Analysis of 'omics data of samples of interest can guide the selection of the most informative readouts by first indicating which cell types are present, second by identifying active pathways and cellular functions, third by highlighting which molecules separate samples based on defined criteria, and fourth by identifying the cellular components that show variable abundance among samples indicative of heterogeneity. This approach has proven to be very powerful when little is known about a set of samples or when the available prior knowledge is biased toward a subset of markers and cellular functions (Perou et al., 2000). Although 'omics methods are insensitive to low-abundance cell populations, mask the heterogeneous abundances of molecules, and are typically destructive of spatial information, they will be especially effective in relating personalized genomic information to tissue phenotype as determined by highly multiplexed imaging methods to enable precision medicine applications (Figure 1).

Alternatively, this workflow could be reversed. Highly multiplexed epitope imaging approaches could be used to study tissues and to demarcate interesting tissue areas. These areas could be characterized with 'omics approaches. Multiple methods to microdissect and isolate cells and tissue areas exist including laser-based cutting (Bonner et al., 1997). Although the collection of tissue areas is tedious using microdissection methods, automated approaches are available to facilitate such analysis. The 'omics data from tissues collected this way could be readily integrated with highly multiplexed epitope imaging data. This approach could allow us to understand whether correlations, such as those between specific microenvironments and clinical outcomes, also reflect altered biological processes (not captured by highly multiplexed epitope imaging). It would also facilitate the study of only morphologically distinct cells (e.g., those becoming invasive and metastatic).

Finally, highly multiplexed epitope imaging could be combined with other mass spectrometry-based imaging methods (Ellis et al., 2014; Schwamborn and Caprioli, 2010). Direct analysis of the tissue with secondary ion mass spectrometry allows analysis of low-mass molecules at subcellular resolution (Steinhauser et al., 2012), whereas matrix-assisted laser desorption ionization methods allow for analysis of high-mass molecules such as proteins at low spatial resolution, typically averaging over many cells per pixel (McDonnell and Heeren, 2007). These measurements yield a broad view of small molecules and proteins; however, due to their untargeted nature, interesting molecules are frequently not detected and many measured ions are unknown. Parallel tissue sections can be analyzed by mass spectrometry-based imaging and highly multiplexed epitope based imaging. These images can then be registered via fiducial markers and integrated with highly multiplexed epitope imaging data to yield a comprehensive view of tissue components. This approach may be suitable when tissue samples are limited, precluding microdissection.

Applications for Precision Medicine and Systems Analysis of Disease

Data generated from tissues using highly multiplexed epitope imaging approaches are complex. In the case of diseases like cancer, the data may seem chaotic and the high dimensionality (measured epitopes and spatial dimensions) typically renders the data hard to visualize and interpret. To analyze the highly multiplexed tissue images, we need computational methods that incorporate cell type, cell state, and spatial information to identify patterns in the data (Figure 1B). Statistical approaches and machine learning methods can be employed to determine if, for example, cell states are found in proximity more often than predicted by chance or if states of neighboring cells correlate with each other. Given that certain phenotypes require that specific cell types and states need to meet in time and space, it is likely that such patterns of cell types, in similar states, in defined spatial arrangements can be repeatedly found. Such patterns, which I will call *tissue motifs*, are exemplified by stem cell niches and are well known in the immune system (e.g., a B cell interacting with a T cell for activation would be a simple tissue motif). The advantage of identification of such tissue motifs is that they often reflect biological processes and readily support an understanding of tissue biology.

Tissue motifs alone are unlikely to capture all systems level information contained in highly multiplexed epitope imaging data, but spatial network modeling is expected to reveal relationships that are not obvious in high-dimensional data (Figure 1B) (Koller and Friedman, 2009). In this context, a "network" describes the dependencies of the analyzed nodes to each other and to spatial features. Generally, network structures are determined by choosing a network model architecture, then systematically estimating model parameters and varying its specific structure, and finally scoring the structures with respect to the fit to the data (Koller and Friedman, 2009). Many network reconstruction approaches exist. Bayesian networks and Markov random fields are two common approaches applied to determine statistical relationships between the measured nodes from single-cell data that reveal the underlying network structure in a probabilistic manner (Kholodenko et al., 2012). These methods have advantages (Koller and Friedman, 2009; Sachs et al., 2005): they are flexible enough to accommodate spatial information (Snijder

et al., 2009) and account for noise that might be present in the data. They enable identification of non-trivial, arbitrarily complex relationships, even if not all of the players were analyzed directly and approaches to control for overfitting are well established (Koller and Friedman, 2009; Sachs et al., 2005).

With such spatial in silico network models, aspects of tissue biology in health and disease that are contained in the data in non-obvious ways can be interrogated. For example, high-dimensional complex dependencies between cell types, the cellular functional state, and neighborhood features can be systematically analyzed, and hypotheses about biological mechanisms or optimal drug perturbations can be generated. From a biologist's point of view, this enables modeling of cell-cell communication and microenvironment's control of cell state. From a biomedical point of view, causes of pathological states and specific phenomena such as tumor cell heterogeneity can be analyzed. If sufficient genomic data are available, genotypic alterations can be related to both network and disease phenotype.

In addition to supporting the study of tissue biology in health and disease, the systems level measurements of highly multiplexed epitope imaging data will enable discovery of novel biomarkers (Figure 1C). Expression-based biomarkers are already routinely used in clinics. For example, breast cancer is classified by assessment of HER2, estrogen receptor, and progesterone receptor levels (Sims et al., 2007). Abnormal expression of these factors drive tumor development, and this dysregulation can be detected via immunohistochemistry and genomic/transcriptomic analyses. Other statistically predictive biomarkers may only become clear when their spatial distribution is observed.

To discover such biomarkers, large cohorts of patient samples with associated personal, clinical, and ideally genomic and transcriptomic data could be compared against data generated by highly multiplexed epitope imaging of the same samples. These cohorts are readily available as formalin-fixed, paraffin-embedded tissue microarrays for most disease types. Typically, the abundances of one or a few epitopes are correlated with clinical data to classify patients and to guide patient care (Sims et al., 2007). However, all levels of highly multiplexed epitope imaging data, including cell types, cell state, cellular environments, and tissue motifs, certainly carry more information for biomarker discovery than routine immunohistochemistry approaches. The mathematical models of the tissues can be expanded to include clinical and genomic features to retrieve non-trivial relationships as biomarkers. Making these biomarkers useful for clinical practice will require either highly multiplexed epitope imaging in clinics or reducing the number of markers needed, with minimal loss of information, to make them amenable to standard immunofluorescence microscopy and immunohistochemistry methods already used in the clinics.

Similarly, highly multiplexed epitope imaging will be a key tool for drug discovery and for assessment of the efficacies and mechanisms of drugs in their target tissue (Figure 1D) (Bodenmiller et al., 2012; Minchinton and Tannock, 2006; Willmann et al., 2008). Tissue motifs that represent the cellular environment could be attractive drug targets (Hanahan and Coussens, 2012). This is exemplified by the recent success of anti-PD1 immunotherapies in melanoma patients to reactivate T cells that were inactivated by cell-cell interaction with tumor and other cells via the PD1 receptor (Pardoll, 2012). Further, the distribution and effect of a drug (assuming it is fluorescent, can be metal labeled, or can be bound by an affinity binder) could be systematically visualized over time in a tissue (Minchinton and Tannock, 2006; Willmann et al., 2008). Such pharmacokinetic analyses are particularly intriguing in cancer, as it is often not known whether a drug is ineffective because it has not reached all tumor cells or because some cells are resistant to the therapy.

Highly multiplexed epitope imaging in conjunction with personal, clinical, and genomic data can be used to accurately diagnose and classify a disease, making it useful in precision medicine and next-generation pathology applications (Figure 1E) (Hood and Friend, 2011; Rimm, 2014). Based on the measurement of the features of the patient tissue sample, an optimal treatment could be selected and an ideal patient care could be chosen. The efficacy of the drug or combination of drugs could, in turn, be assessed by highly multiplexed imaging and treatment adjusted if necessary. Expanding the repertoire of highly multiplexed epitope imaging to measurements of DNA, RNA, enzymatic activity, and metabolites and use in combination with complementary 'omics data, as discussed above, promises to make highly multiplexed epitope imaging an integrative center part of clinical diagnosis and precision medical applications in the future.

Conclusions

Different approaches for highly multiplexed epitope imaging, each with strengths and weaknesses, are under development, and the field is advancing rapidly. Highly multiplexed epitope imaging has the potential to transform the way we study tissue biology in health and disease and how we assess clinical samples. We need to begin planning now to ensure that data are comparable through time and across laboratories and hospitals (Table 2). Highly multiplexed epitope imaging is poised to broadly complement immunofluorescence microscopy in life science and pharmaceutical research and will become central to precision medical applications in the clinics. Exciting times are ahead.

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